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Original Article

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T cell cytokine synthesis at the single-cell level in BALB/c and C57BL/6 mice infected with ectromelia virus*

Synteza cytokin na poziomie komórkowym przez limfocyty T u myszy BALB/c i C57BL/6 zakażonych wirusem ektromelii

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The purpose of the study was to evaluate synthesis of IFN-γ, IL-2, TNF-α (Th1/Tc1) and IL-4 (Th2/Tc2) at $CD4^+$ T and $CD8^+$ T cell level in BALB/c and C57BL/6 mice in the course of infection with ectromelia virus Moscow strain (ECTV-MOS).

Material/Methods: Synthesis of IFN-γ, IL-2, TNF-α and IL-4 in $CD4^+$ T and $CD8^+$ T cells in draining lymph nodes (DLNs) and spleens of BALB/c and C57BL/6 mice was detected by intracellular staining and flow cytometry analysis.

Results: Our results showed an increase in percentage of IFN-γ-synthesizing $CD8^+$ T cells only in DLNs and spleens of C57BL/6 mice at the early stages of infection. Moreover, synthesis of IL-2 by $CD4^+$ and $CD8^+$ T cells occurred earlier and was stronger in C57BL/6 mice compared to BALB/c mice. The increase in TNF-α synthesis by $CD4^+$ T and $CD8^+$ T cells was detected mainly in DLNs of infected animals. We did not observe any changes in the percentage of IL-4-synthesizing T cells (Th2 and Tc2) during ECTV-MOS infection in both strains of mice.

Conclusions: Results presented in this study confirmed that during the early phase of infection, C57BL/6 mice mounted a strong Th1 and Tc1 immune response against ECTV-MOS. BALB/c mice that survived the acute stage of mousepox, were able to mount an adequate cellular response to ECTV-MOS, however successful elimination of the virus in susceptible mice may occur more slowly compared to resistant strains of mice. Intracellular detection of IL-4 by flow cytometry was not sensitive enough to distinguish the differences in IL-4-synthesizing Th2 and Tc2 cells between susceptible and resistant strains of mice during ECTV-MOS infection.

Key words: ectromelia virus • mousepox • cytokines • T lymphocytes

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Streszczenie

Wstęp: Przedmiotem pracy było określenie syntezy IFN-γ, IL-2, TNF-α (Th1/Tc1) oraz IL-4 (Th2/Tc2) na poziomie pojedynczych limfocytów T CD4+ i T CD8+ w przebiegu zakażenia myszy BALB/c i C57BL/6 szczepem Moscow wirusa ektromelii (ECTV-MOS).

Materiał/Metody: Syntezę IFN-γ, IL-2, TNF-α oraz IL-4 przez limfocyty T CD4+ i T CD8+ w drenujących węzłach chłonnych (DLN) i śledzionach myszy BALB/c i C57BL/6 oceniano metodą barwienia wewnątrzkomórkowego z analizą cytometryczną.

Wyniki: Przedstawione wyniki wskazują na wzrost odsetka limfocytów T CD8+ syntetyzujących IFN-γ we wczesnej fazie zakażenia ECTV-MOS jedynie w DLN i śledzionie myszy C57BL/6. Ponadto synteza IL-2 przez limfocyty T CD4+ i T CD8+ wystąpiła u tych zwierząt wcześniej i była silniejsza w porównaniu do myszy BALB/c. Wzrost syntezy TNF-α przez limfocyty T CD4+ i T CD8+ obserwowano zwłaszcza w DLN zakażonych zwierząt. Nie wykazano różnic w odsetku limfocytów T syntetyzujących IL-4 (Th2 i Tc2) u obu szczepów myszy podczas zakażenia ECTV-MOS.

Wnioski: Przedstawione wyniki potwierdziły, że myszy C57BL/6 rozwijają silną odpowiedź Th1 oraz Tc1 we wczesnej fazie zakażenia ECTV-MOS. Myszy BALB/c, które przeżyły ostrą postać choroby, były również w stanie wykształcić dostatecznie silną odpowiedź komórkową przeciwko ECTV-MOS, jednak skuteczna eliminacja wirusa u wrażliwych szczepów myszy może następować wolniej niż u myszy C57BL/6. Niewielka czułość metody wewnątrzkomórkowego wykrywania IL-4 za pomocą cytometrii przepływowej nie pozwoliła na ocenię różnic w syntezie IL-4 przez komórki Th2 i Tc2 między wrażliwymi a opornymi szczepami myszy podczas zakażenia ECTV-MOS.

Słowa kluczowe: **wirus ektromelii • ospa myszy • cytokiny • limfocyty T**

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Introduction

Activation of T cells results in production of cytokines that play a crucial role in orchestrating the immune response required to control and clear invading pathogens. Differences in cytokine secretion profiles, expression of cell surface chemokine receptors and nuclear transcription factors are exhibited by various subpopulations of CD4+ T cells exposed to different environmental stimuli [7,16,17,27,31]. The type of T helper (Th) immune response determines the onset of many viral infections. Early production of cytokines such as interferon (IFN)-α/β, IFN-γ, interleukin (IL)-2 and IL-12p40 induces a strong cellular immune response, that is critical for virus clearance and full recovery (profile associated with Th1 cells). On the other hand, in many viral infections generation of Th2 immune response, associated with IL-4, IL-5, IL-10 and IL-13 production, does not provide protection and often leads to a progressive and fatal disease [18,19].

Similar to CD4+ T cells, cytotoxic CD8+ T lymphocytes (CTLs) can be classified into at least two distinct subsets based on their cytokine-secreting profiles and chemokine receptors [22]. Type 1 CD8+ T cells (Tc1) predominantly secrete IFN-γ, IL-2 and tumor necrosis factor (TNF)-α, whereas Tc2 effector cells preferentially secrete IL-4, IL-5, IL-6 and IL-10 [1,22]. Tc1 cells have a capacity to migrate to inflamed tissues and kill tumor or virus-infected cells [12]. Tc2 cells were shown to have a reduced antiviral activity *in vivo* not due to reduced secretion of IFN-γ, but due to alteration in homing to the target organ [30]. The presence of Tc2 cells often correlates with disease severity and progression in chronic viral infections, cancer, and neurologic and autoimmune diseases [12]. The importance of Tc2 cells in progression of chronic pathologies may be explained by their capacity to modify the function of dendritic cells (DCs) and favor differentiation of naïve CD4+ and CD8+ T cells to type 2 cells [12].

Alteration of the Th1/Th2 and Tc1/Tc2 balance is observed in many pathological conditions including immune-mediated inflammatory diseases such as multiple sclerosis, rheumatoid arthritis, progressive systemic sclerosis, systemic lupus erythematosus, and chronic obstructive pulmonary disease [15,29]. In many viral and bacterial infections generation of inappropriate type of immune response can exacerbate disease and lead to inefficient elimination of the infectious agent.

Ectromelia virus (ECTV) is a member of the *Poxviridae* family, genus *Orthopoxvirus* and is a causative agent of mousepox, a disease called "smallpox of mice". ECTV is closely related to variola virus (VARV) – a causative agent of smallpox responsible for millions of deaths in the history of mankind. Mousepox model is arguably the most suitable small animal model for the study of orthopoxvirus infections including smallpox. Moreover, mousepox model is used extensively to investigate the pathogenesis of generalized viral infections, genetic resistance to disease and viral immunology [2,3,4,13,20,25].

Among various inbred strains of mice there is genetically determined susceptibility to lethal infection with ECTV. Thus, BALB/c, DBA (both H-2^d haplotype) and C3H (H-2^k) mice have been classified as genetically susceptible, while C57BL/6, C57BL/10 and 129/Sv (all H-2^b) mice are resistant to lethal infection with ECTV [2]. Strain variations in resistance to lethal form of mousepox are controlled by multiple, unlinked, dominant genes, including the natural killer (NK) cell genes complex, the fifth component of complement (C5) gene, major histocompatibility complex (MHC) genes and selectin gene complex [5,6]. Resistant strains of mice recover from primary ECTV infection due to mobilization of multiple mechanisms of innate and adaptive immune response, including production of IFNs, activity of NK cells and macrophages (Mø), activation of complement system, early and strong activation of virus-specific CTLs and production of antiviral antibodies (Abs) [3,13,20].

Chaudhri and colleagues [4] have shown that during ECTV infection, resistant mice produce high level of Th1 cytokines, such as IFN-γ, IL-2 and TNF-α, which was associated with strong cellular immune response and full recovery. On the contrary, in susceptible strains of mice the production of Th1 cytokines was absent or at a very low level, leading to weak and delayed cellular response resulting in high mortality rates [4]. However, the production of Th1/Th2 and Tc1/Tc2 cytokines by $CD4^+$ and $CD8^+$ T cells, respectively, during mousepox has not been fully investigated. Thus, the purpose of the present study was to evaluate the synthesis of IFN-γ, IL-2, TNF-α (Th1/Tc1) and IL-4 (Th2/Tc2) by $CD4^+$ and $CD8^+$ T cells in BALB/c and C57BL/6 mice during primary infection with Moscow strain of ECTV (ECTV-MOS).

Materials and Methods

Animals

Inbred, male BALB/c (H-2^d) and C57BL/6 (H-2^b) mice, between 8–12 weeks of age, were purchased from the Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology in Warsaw, Poland. Animals were acclimated for 1 week before experiments and were allowed *ad libitum* access to food and water. Mice used in studies described in this report were sacrificed by cervical dislocation. All experiments were conducted under the permission granted by the 3rd Local Ethical Commission for Animal Research at the Warsaw University of Life Sciences-SGGW (No. 61/2009).

Virus

Highly infectious ECTV-MOS (ATCC 1374) was grown in Vero cells (ATCC CCL-81) monolayer, then titrated and stored in aliquots at –80°C until used. Fifty plaque forming units (PFU) of ECTV-MOS were injected into the hind foot pad (f.p.) in a total volume of 20 µl/f.p of an anesthetized mouse. BALB/c and C57BL/6 mice injected with sterile phosphate-buffered saline (PBS) served as negative controls.

Antibodies

The following monoclonal antibodies (mAbs) directed against mouse leukocyte surface markers and mouse intracellular cytokines in appropriate combinations were used: anti-CD4-PerCP (H129.9), anti-CD8-PE or anti-CD8-FITC (53-6.7), anti-IFN-γ-PE (XMG1.2), anti-IL-2-PE (JES6-5H4), anti-TNF-α-FITC (MP6-XT22) and anti-IL-4-FITC (11B11). The antibodies were initially titrated to obtain the optimal concentration. All antibodies and appropriate isotype controls were purchased from BD Biosciences.

Reagents

Phorbol 12-myristate 13-acetate (PMA) (Sigma) and ionomycin (Sigma) were used for nonspecific stimulation of cytokine production. GolgiPlug reagent (containing brefeldin A that has an inhibitory effect on protein secretion) and reagents for cell fixation and permeabilization (Cytofix/Cytoperm and Perm/Wash) were obtained from BD Biosciences.

Preparation of single cell suspension

Draining lymph nodes (DLNs – popliteal and superficial inguinal) and spleens were aseptically removed at 7, 14 and 21 day post infection (d.p.i.) or from uninfected control animals. The number of animals per experimental group was as follows: control BALB/c mice (n=5 to 8); control C57BL/6 mice (n=5 to 9); BALB/c mice at 7 d.p.i. (n=6 to 11); C57BL/6 mice at 7 d.p.i. (n=5 to 6); BALB/c mice at 14 d.p.i. (n=5 to 6); C57BL/6 mice at 14 d.p.i. (n=5 to 11); BALB/c mice at 21 d.p.i. (n=5 to 6); C57BL/6 mice at 21 d.p.i. (n=5 to 10). The isolated organs were mechanically disaggregated by passing through stainless steel sieves. After erythrocyte depletion the viability of cells was assessed using 0.4% trypan blue exclusion test.

Enumeration of cytokine-synthesizing cells by intracellular staining

Freshly isolated cells were restimulated *in vitro* with ionomycin/PMA in accordance with BD Biosciences protocols and literature data [8,9]. Briefly, cells were placed in 96-well plates and stimulated using PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 µg/ml) for 4 hrs at 37ºC in 5%CO_2. Later, cells were washed in PBS supplemented with 3% fetal bovine serum (FBS) and blocked for nonspecific binding in 30% FBS for 30 min on ice. Surface staining was then performed using mAbs anti-CD4 and anti-CD8 followed by intracellular staining with Cytofix/Cytoperm kit in accordance with the manufacturer's instructions. Briefly, cells were fixed and permeabilized with Cytofix/Cytoperm solution for 25 min on ice followed by washing in Perm/Wash solution. Next, cells were stained for 30 min on ice with mAbs against intracellular cytokines: IFN-γ, IL-2, TNF-α or IL-4 and finally

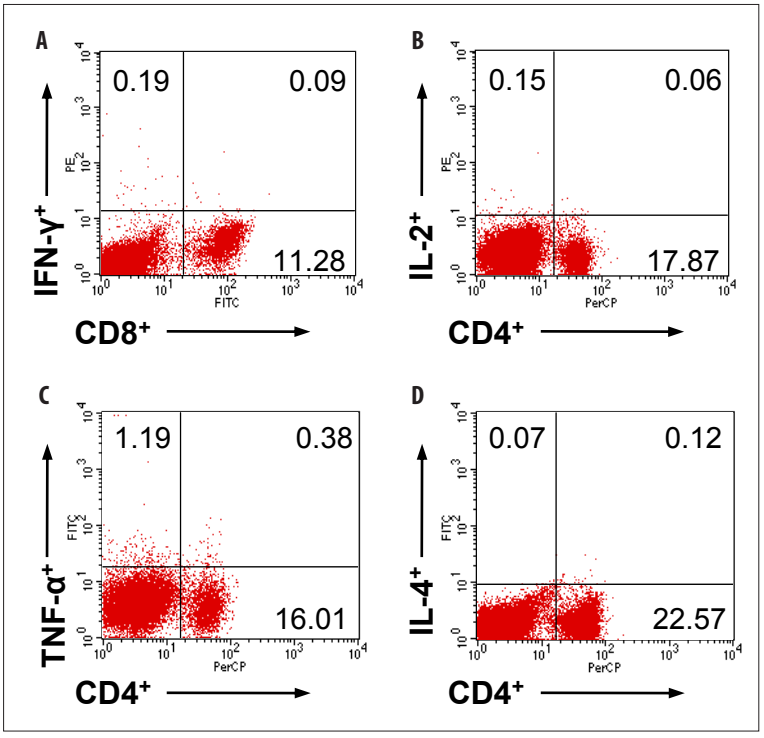

Fig. 1. Representative dot plots show spontaneous synthesis of IFN-γ (**A**) by CD8+ T cells and IL-2 (**B**), TNF-α (**C**) and IL-4 (**D**) by CD4+ T cells isolated from spleens of uninfected BALB/c (**D**) or C57BL/6 (**A, B, C**) mice. Cells were incubated for 4 hrs in the presence of brefeldin A. Numbers within quadrants represent the percentage of positive cells for a given marker within the gate for lymphocyte population defined by FSC and SSC parameters

washed in Perm/Wash solution and 3% FBS/PBS, fixed in 2% paraformaldehyde (PFA) in PBS and analyzed within 24 hrs by flow cytometry.

Flow cytometry acquisition and analysis

Percentage of cytokine-synthesizing cells was measured by FACSCalibur flow cytometer (Becton Dickinson) and analyzed by CellQuest software. Data from 2×10^4 and 5×10^4 events were acquired for each sample. Nonlymphoid and dead cells were excluded by appropriate gating based on forward (FSC) and side (SSC) scatter profiles. Single-cell cytokine synthesis was assessed in a gate set on lymphocyte population using FSC and SSC characteristics.

Statistical analysis

Percentage of cytokine-synthesizing CD4+ and CD8+ T cells was given as mean ±SD (standard deviation). Differences between uninfected control mice and infected animals at indicated time points were calculated using the Student's t-test (in case of normal data distribution with equal variance) or Mann-Whitney U-test (STATISTICA 6.0 software, StatSoft). Statistical significance was determined at $P<0.05$.

Results

We examined the synthesis of IFN-γ, IL-2, TNF-α and IL-4 by CD4+ and CD8+ T cells in both susceptible (BALB/c) and resistant (C57BL/6) inbred strains of mice during ECTV-MOS infection. 4 hrs were chosen as an optimal time to achieve synthesis of cytokines in *in vitro* restimulation of isolated cells with ionomycin and PMA (data not shown). Unstimulated cells incubated *in vitro* for 4 hrs in the presence of transport inhibitor (brefeldin A) served as a control of spontaneous cytokine synthesis (Figure 1).

Synthesis of IFN-γ by CD4+ and CD8+ T cells in C57BL/6 mice during ECTV-MOS infection

Intracellular detection of IFN-γ in T lymphocytes of BALB/c mice in the early stages of mousepox (until 14 d.p.i.) has been previously described [25]. For that reason only results concerning the synthesis of IFN-γ by T cells in DLNs and spleens of C57BL/6 mice infected with ECTV-MOS are reported here.

During ECTV-MOS infection of C57BL/6 mice, higher percentage of CD8+IFN-γ+ T cells than CD4+IFN-γ+ T cells was observed (Figure 2). In DLNs and spleens of C57BL/6 mice at 7 d.p.i. a statistically significant ($P<0.05$) increase in percentage of CD8+ T cells synthesizing IFN-γ was observed compared to uninfected control animals (18% *vs* 14% and 30% *vs* 17%, respectively) (Figure 2B).

The strongest synthesis of IFN-γ by CD4+ and CD8+ T cells was detected at 14 d.p.i. in both tissues. Moreover, at 14 d.p.i. there was a significantly higher ($P<0.05$) percentage of CD4+IFN-γ+ T cells in DLNs and CD8+IFN-γ+ T cells in DLNs and spleens of C57BL/6 mice compared to BALB/c mice [25]. The percentage of CD8+ T cells synthesizing IFN-γ in DLNs and spleens of C57BL/6 mice started to decrease during recovery, whereas in BALB/c mice it remained high until day 21 p.i. (data not shown).

Differences in percentage of IL-2-synthesizing T cells between BALB/c and C57BL/6 mice during ECTV-MOS infection

During early stages of mousepox (7 d.p.i.) the percentage of IL-2-synthesizing CD4+ T cells in DLNs and spleens of BALB/c mice was lower and their appearance was delayed compared to the C57BL/6 group (Figure 3A, 3C). Increase in the percentage of IL-2-synthesizing CD4+ T cells in DLNs

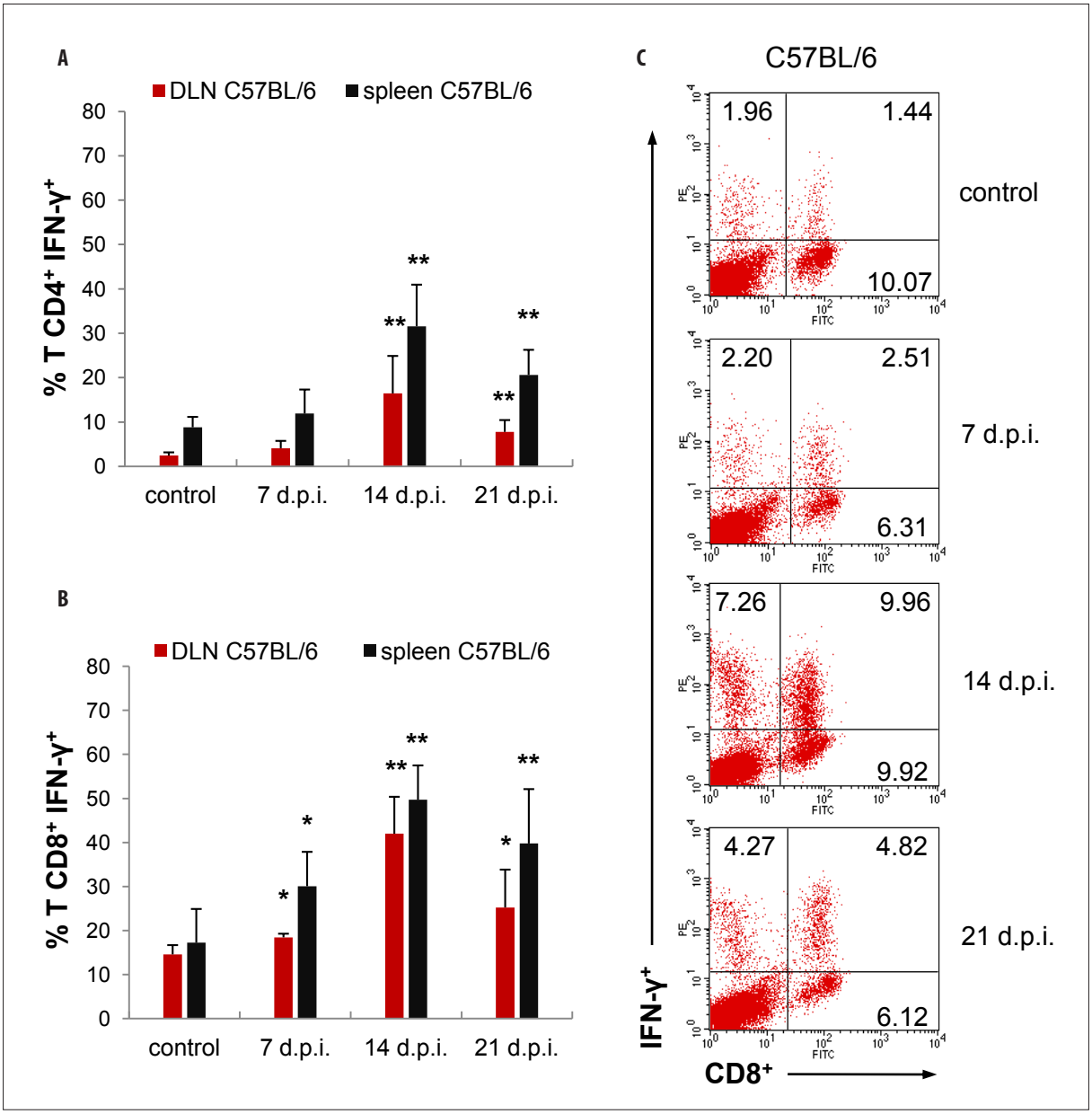

Fig. 2. Percentage of IFN-γ-synthesizing CD4+ (**A**) and CD8+ (**B**) T cells in DLNs and spleens of C57BL/6 mice during mousepox. (**C**) Representative dot plots show synthesis of IFN-γ by CD8+ T cells in spleens of C57BL/6 mice during ECTV-MOS infection. Numbers within quadrants represent the percentage of positive cells for a given marker within the gate for lymphocytes (* P≤0.05, ** P≤0.01)

and spleens of both strains of mice peaked on day 14 p.i. and remained high until day 21 in spleens of infected animals. Meanwhile, the percentage of IL-2-synthesizing CD8+ T cells was approximately 2-fold lower than that of CD4+ T cells during ECTV-MOS infection (Figure 3B). The percentage of IL-2-synthesizing CD8+ T cells varied between the two inbred strains of mice. In DLNs and spleens of BALB/c mice the highest percentage of CD8+IL-2+ T cells was observed at 14 d.p.i. and remained high until 21 d.p.i., whereas in C57BL/6 a statistically significant (P<0.05) increase in percentage of IL-2-synthesizing CD8+ T cells was observed as early as day 7 p.i. and thereafter decreased gradually. In spleens of BALB/c mice at day 14 and 21 p.i., higher percentage of IL-2+CD8+ T cells was observed compared to C57BL/6 mice (Figure 3). High synthesis of this Tc1 cytokine in BALB/c mice continued until day 21 p.i. (Figure 3B).

BALB/c and C57BL/6 mice displayed similar percentage of TNF-α-synthesizing T cells during ECTV infection

In DLNs and spleens of BALB/c and C57BL/6 mice we found similar percentage of TNF-α-synthesizing T cells throughout the course of infection (Figure 4). The percentage of TNF-α-synthesizing CD4+ and CD8+ T cells remained unchanged until 7 d.p.i. in both tissues in BALB/c and C57BL/6 mice. In DLNs of BALB/c and C57BL/6 mice at 14 d.p.i. a statistically significant (P<0.01) increase in TNF-α-synthesizing CD4+ T cells was observed compared to uninfected animals (67% *vs* 38% and 60% *vs* 32%, respectively) (Figure 4A). In spleens, at this time point of infection a significant (P<0.05) increase in TNF-α-synthesizing CD4+ T cells was also detected. However the increases were

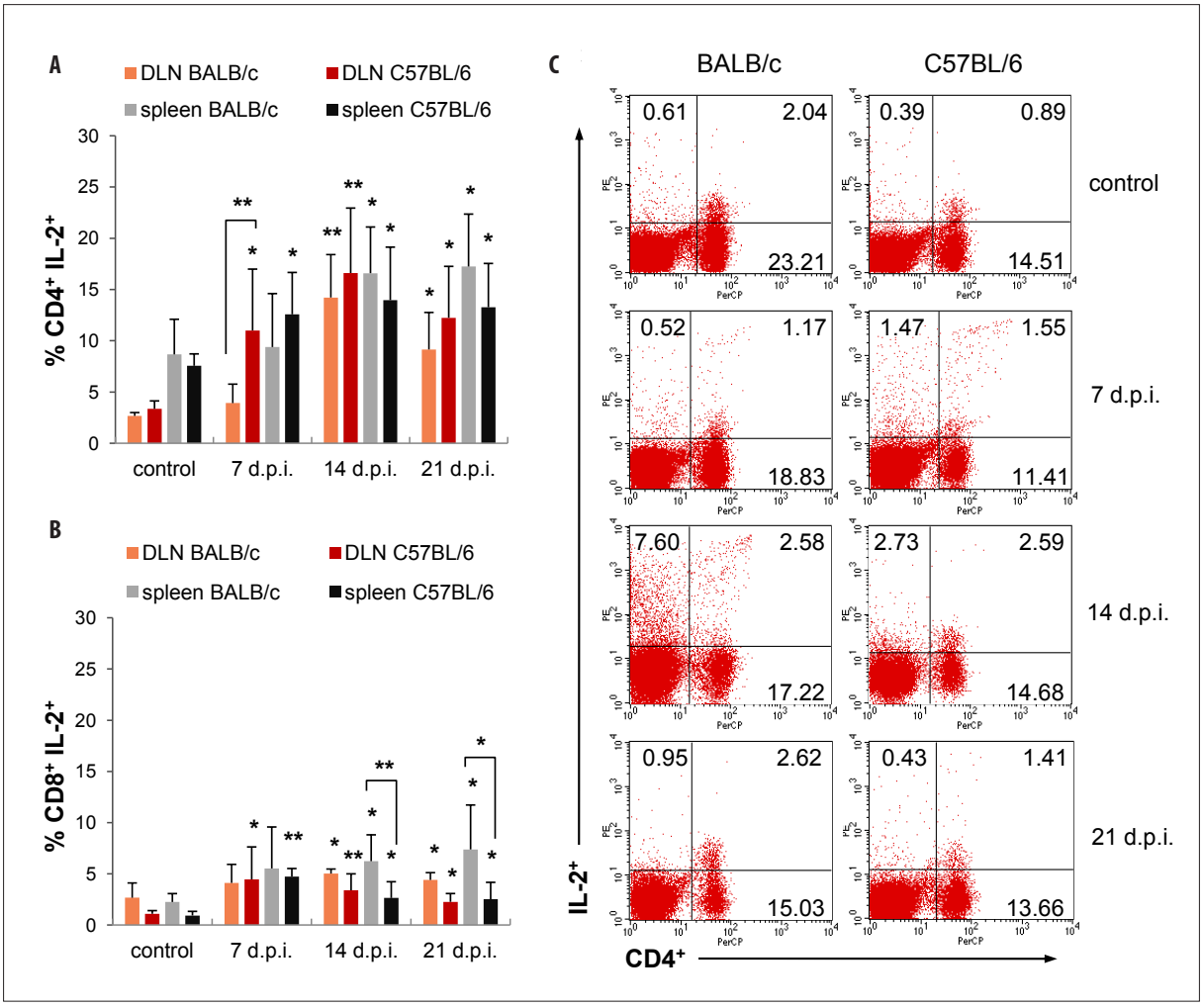

Fig. 3. Percentage of IL-2-synthesizing CD4+ (**A**) and CD8+ (**B**) T cells in DLNs and spleens of BALB/c and C57BL/6 mice during mousepox. (**C**) Representative dot plots show synthesis of IL-2 by CD4+ T cells in spleens of BALB/c and C57BL/6 mice during ECTV-MOS infection. Numbers within quadrants represent the percentage of positive cells for a given marker within the gate for lymphocytes (* P≤0.05, ** P≤0.01)

smaller than those found in DLNs (43% *vs* 32% in BALB/c and 54% *vs* 40% in C57BL/6 mice, respectively). The percentage of CD4+ T cells synthesizing TNF-α in DLNs and spleens of BALB/c and C57BL/6 mice remained high until 21 d.p.i. In both organs of the two strains of mice a statistically significant (P<0.01) increase in TNF-α-synthesizing CD8+ T cells was observed at 14 d.p.i. compared to uninfected animals (Figure 4B). In DLNs the percentage of TNF-α+CD8+ T cells declined by day 21 p.i., whereas in spleens of BALB/c mice it peaked on day 21 p.i.

Flow cytometric analysis did not reveal changes in intracellular IL-4 production by T cells during mousepox

Although CD4+ T cells were the main subpopulation of T cells synthesizing IL-4, during ECTV-MOS infection of BALB/c and C57BL/6 mice no statistically significant changes in the percentage of IL-4-synthesizing CD4+ and CD8+ T cells were detected in DLNs and spleens compared to uninfected group of animals (Figure 5). Moreover, in both T cell subsets no statistically significant changes in mean fluorescence intensity (MFI) of IL-4 expression were observed between control and infected BALB/c and C57BL/6 mice (data not shown).

Discussion

Cytokine production by the responding lymphocytes directs the development of antigen-specific immune response against the infectious agent. The secretion of various cytokines may result in protective cellular immune response but on the other hand may facilitate virus dissemination by suppression of the mechanisms intended to eliminate the infectious agent. In the present study we evaluated the synthesis of Th1/Th2 and Tc1/Tc2 cytokines by CD4+ and CD8+ T cells, respectively, in both susceptible (BALB/c) and resistant (C57BL/6) strains of mice infected with highly virulent ECTV-MOS. For single-cell level evaluation of cytokine synthesis we used intracellular staining technique followed by flow cytometric analysis.

Our results indicated that there are distinct differences in the percentage of Th1/Tc1 cytokine-synthesizing cells in resistant and susceptible mice during mousepox (Figures 2–4). In C57BL/6 mice the synthesis of Th1/Tc1 cytokines (IFN-γ and IL-2) occurred earlier than in BALB/c mice [25]. This may reflect strong proliferation of CD4+ and CD8+ T cells following antigen specific recognition in C57BL/6 mice, and in consequence, T cells migrate to effector sites such as

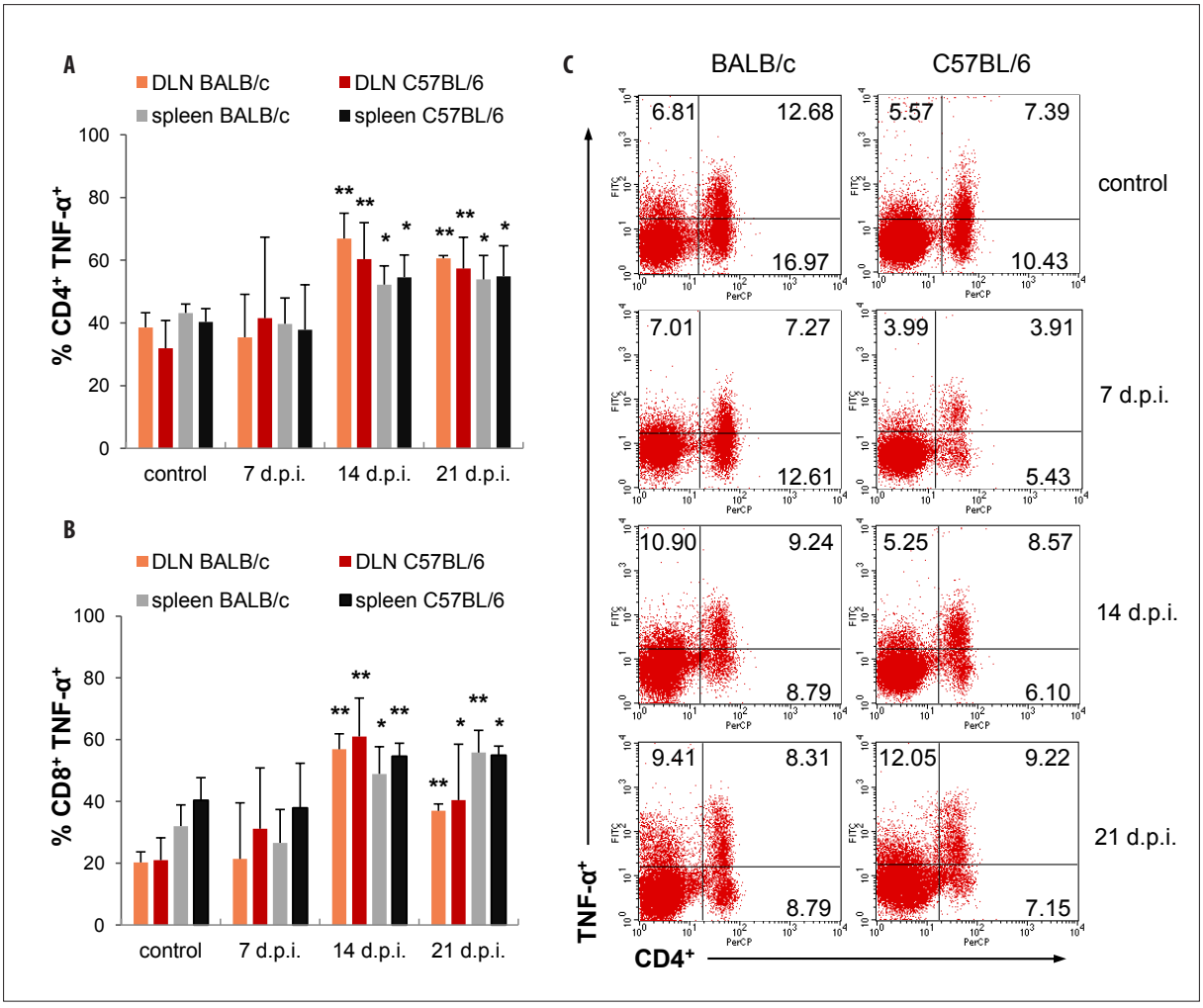

Fig. 4. Percentage of TNF-α-synthesizing CD4+ (**A**) and CD8+ (**B**) T cells in DLNs and spleens of BALB/c and C57BL/6 mice during mousepox. (**C**) Representative dot plots show synthesis of TNF-α by CD4+ T cells in spleens of BALB/c and C57BL/6 mice during ECTV-MOS infection. Numbers within quadrants represent the percentage of positive cells for a given marker within the gate for lymphocytes (* P≤0.05, ** P≤0.01)

the spleen, liver and other organs for virus elimination [4]. Moreover, during secondary infection with ECTV, DLNs are the sites where lymphocytes become activated and proliferate, and where infected cells are eliminated by memory CD8+ T cells acting as gatekeepers that directly kill targets infected with ECTV [32]. Our results confirmed that anti-ECTV--MOS defense mechanisms at the early stages of infection come into effect much earlier in resistant strains of mice such as C57BL/6 whereas BALB/c mice display a delay in generation of the optimal antiviral cellular immune response [18,23].

Besides the differences between strains of mice concerning the synthesis of Th1/Tc1 cytokines in the initial phase of infection, the kinetics of its synthesis at later stages of infection showed certain similarities (Figures 2–4). In both strains of mice the greatest synthesis of IFN-γ, IL-2 and TNF-α by CD4+ and CD8+ T cells in DLNs and spleens was observed at 14 d.p.i., when the clinical signs of mousepox were severe. IFN-γ was synthesized mainly by CD8+ T cells, IL-2 mainly by CD4+ T cells, whereas TNF-α was synthesized by both subsets of T cells at comparable levels. Our data on cytokine synthesis support the notion that ECTV-MOS-specific CD8+ and CD4+ CTLs form the basis of cellular immune response at 14 d.p.i. in infected BALB/c [23] and C57BL/6 mice. However, susceptible BALB/c mice generated weaker Th1/Tc1 immune response than resistant C57BL/6 mice, which indeed led to recovery from mousepox, but could not be sufficient for complete elimination of viral particles in the infected host. It has been shown that ECTV causes a persistent infection in splenic DCs and Mø of BALB/c mice following the acute infection [24]. Meantime IFN-γ is the major cytokine that activates Mø, increasing their activity to kill intracellular pathogens and viruses. Moreover, the activity of IFN-γ can be inhibited at the level of receptor engagement by soluble IFN-γR homolog (vIFN-γR), a viroceptor encoded by ECTV, which represents a strategy to evade the antiviral effects of this cytokine [6]. High production of Th1/Tc1 cytokine until 21 d.p.i. in BALB/c mice suggests that in susceptible strains of mice the successful elimination of the virus from the infected host through adaptive immune response occurs more slowly than in resistant mice.

IFN-γ is a critical effector cytokine responsible for ECTV elimination from all major organs, except the primary sites of infection, where viral clearance appears to be delayed [14]. Moreover, IFN-γ produced by the CD4+ helper T cells is involved in the generation of optimal CD8+ CTLs response [18]. The importance of IFN-γ in the development

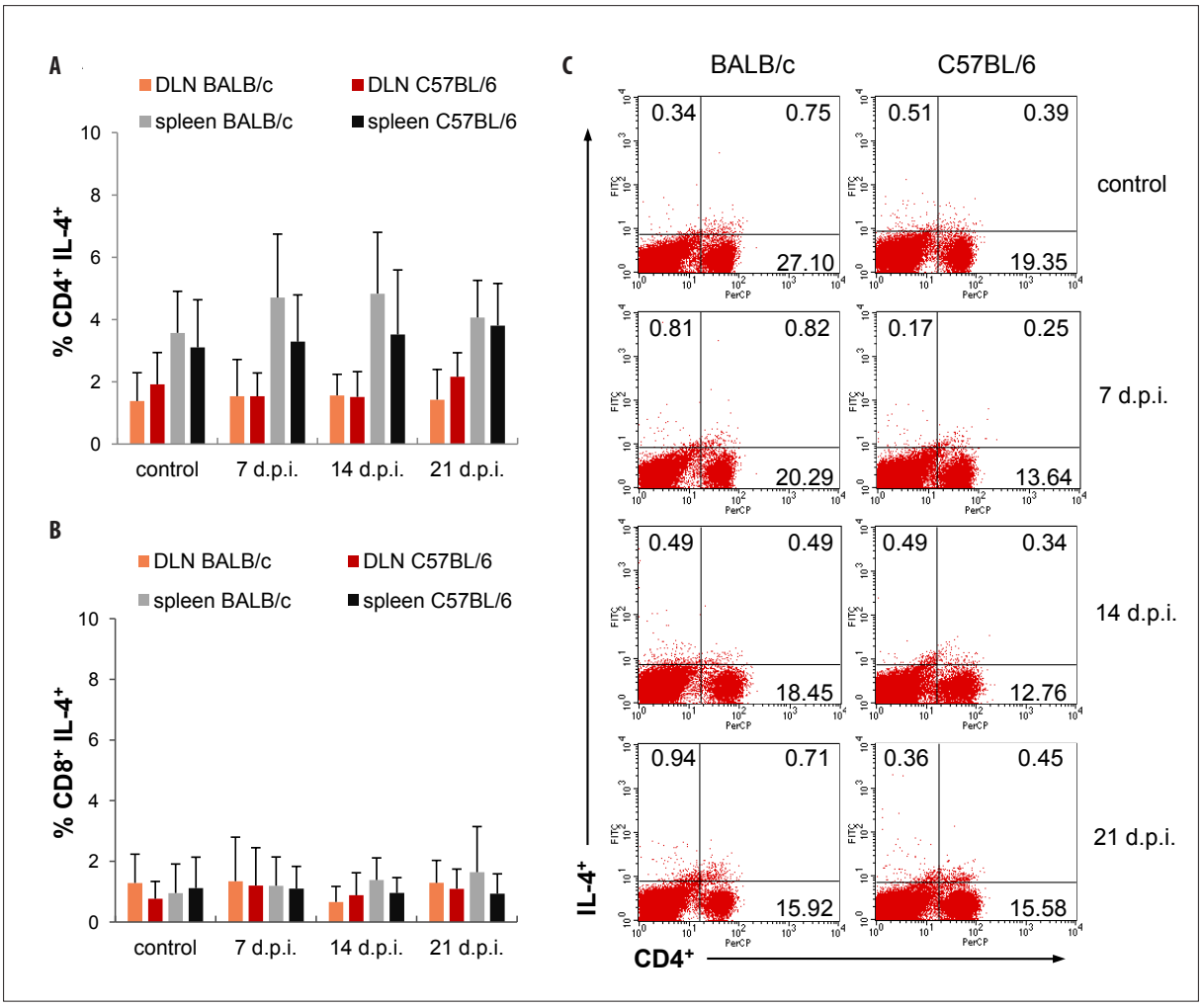

Fig. 5. Percentage of IL-4-synthesizing CD4+ (**A**) and CD8+ (**B**) T cells in DLNs and spleens of BALB/c and C57BL/6 mice during mousepox. (**C**) Representative dot plots show synthesis of TNF-α by CD4+ T cells in spleens of BALB/c and C57BL/6 mice during ECTV-MOS infection. Numbers within quadrants represent the percentage of positive cells for a given marker within the gate for lymphocytes (* P≤0.05, **P≤0.01)

of Th1 cytokine immune response and elimination of the virus has been shown using neutralizing mAbs. Treatment of C57BL/6 mice with neutralizing mAbs against IFN-γ resulted in enhanced spread to and efficient ECTV replication in the spleen, lungs, ovaries and liver, transforming infection from a self-limiting disease to fulminant mousepox [4,14].

It has been reported that neutralizing TNF-α or IL-2 function in C57BL/6 mice does not significantly affect viral replication and does not reverse the resistance to mousepox [4]. However, normal or immunodeficient mice infected with vaccinia virus (VACV) expressing IFN-γ or TNF-α revealed attenuation of virus-induced pathogenicity and rapid virus clearance [11]. It has been shown, that the antiviral effect of TNF-α on ECTV depends on p55 and p75 TNF receptors. Mice lacking p75 were significantly more susceptible to ECTV and most mice succumbed to lethal infection [21]. TNF-α synergizes with IFN-γ to induce multiple antiviral activities. Intriguingly, TNF-α production is cell-type restricted and cannot be substituted by other cellular sources. TNF-α produced by antigen-specific CD4+ and CD8+ T cells is involved in direct killing of infected cells and activation of inflammatory response [10]. In our studies, higher synthesis of TNF-α by T cells in DLNs of infected animals confirms that TNF-α secretion is organ specific and may represent unique requirements for distinct cytokines in T cell priming [4].

Intracellular detection of IL-4 did not reveal any statistically significant changes in the percentage of IL-4-synthesizing CD4+ (Th2) and CD8+ (Tc2) T cells during mousepox in both strains of mice (Figure 5). It is not excluded that flow cytometric analysis of intracellular IL-4 is not sufficient for this purpose due to its low sensitivity [28]. Meanwhile, it has been shown that susceptible strains of mice generate a Th2 cytokine response with IL-4 production [4], however the cellular origin of IL-4 has not been defined. It is known that IL-4 down-regulate the production of Th1 cytokines. This may explain why generation of IL-2 and IFN-γ is delayed at the early stages of mousepox in BALB/c mice compared to C57BL/6 mice. On the other hand it was shown, that the absence of IL-4 did not reverse the susceptibility of BALB/c mice to ECTV infection [4]. The immune responses to ECTV by both IL-4 gene knockout mice on a BALB/c background and BALB/c mice treated with neutralizing mAbs against IL-4 were similar to wild type BALB/c mice [4]. Moreover, inactivation of signal transducer and activator of transcription 6 (STAT6), which is responsible

for IL-4-mediated differentiation events, was not sufficient for BALB/c mice to overcome ECTV infection. However, STAT6$^{-/-}$ mice infected with ECTV displayed minimal lesions in the vital organs and delayed mortality, what correlated with suppressed Th2 immunity [18]. Chaudhri and colleagues [4] have shown that despite the differences in IL-4, IL-2, TNF-α and IFN-γ production in DLNs and spleens between different strains of mice, the expression of mRNA for those cytokines was comparable between BALB/c and C57BL/6 mice suggesting the existence of posttranscriptional regulation of cytokine expression *in vivo*.

Recently, we have found that during early stages of mousepox in spleens of BALB/c mice there was an increase in IL-17A-producing CD4$^+$ and CD8$^+$ T cells [26]. Therefore, IL-17A may play a role in both elimination of viral particles or promoting the tissue damage during the acute phase of infection with ECTV-MOS [26].

In conclusion, our results confirmed that at early stages of ECTV-MOS infection, C57BL/6 mice mounted earlier and stronger type 1 cytokine response. When infected with small dose of virus (50 PFU) BALB/c mice were able to survive an acute phase of infection and were able to mount relatively strong and effective Th1/Tc1 immune response. In most cases, however, anti-ECTV-MOS defense mechanisms at the early stages of infection came into effect too late and for those reasons susceptible strains of mice often succumbed to a disease.

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